



Genome-wide association studies unravel genomic regions and candidate genes associated with *Aspergillus flavus* resistance in peanut kernels

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Key message In the present study, we identified 10 significant single nucleotide polymorphisms (SNPs), 3 stable quantitative trait loci (QTLs) and 3 potential candidate genes associated with peanut kernel resistance to *Aspergillus flavus* stress.

Abstract Peanut (*Arachis hypogaea* L.) is highly susceptible to *A. flavus* infection, producing highly carcinogenic aflatoxins. Breeding resistant varieties is an effective and sustainable approach to address this issue, and identifying novel genetic sources and loci underlying resistance is crucial. In this study, 353 *A. hypogaea* accessions were evaluated for resistance to *A. flavus* infection and aflatoxin production across three environments, leading to the identification of 13 accessions with stable resistance to both infection and aflatoxin production. A genome-wide association study (GWAS) was performed by integrating phenotypic data from multiple environments with 935,231 SNP markers, resulting in the detection of 10 significant marker-trait associations (MTAs). Of these, one showed consistent association with infection resistance in at least two out of three environments, and two were associated with resistance to both aflatoxin B₁ (AFB₁) and aflatoxin B₂ (AFB₂) production. Based on linkage disequilibrium (LD) decay estimates (70 kb upstream and downstream of significant SNPs), three novel stable QTLs were identified on chromosomes A03 and A05, designated *qII_A05*, *qAF_A03*, and *qAF_A05*, and 13 candidate genes were initially selected within these QTL regions. Haplotype analysis further validated 3 key candidate genes: *Arahy.7046BI.1* (encoding a PH/START domain-containing protein), *Arahy.TX2FLU.1* (encoding a receptor-like kinase), and *Arahy.ASR4JM.1* (encoding a TIR-NBS-LRR class disease resistance protein). These genes exhibited significant haplotype-trait associations, with favorable haplotypes showing high frequencies (85.43%–94.12%) in the germplasm pool, facilitating their direct utilization in breeding. Overall, this study uncovers key genetic loci and candidate genes governing peanut resistance to *A. flavus* infection and aflatoxin production, provides elite resistant germplasm resources, and offers practical insights for marker-assisted selection. The findings lay a solid foundation for accelerating the development of *A. flavus*-resistant peanut varieties and provide valuable genetic information for peanut breeding programs.

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Introduction

Peanut (*Arachis hypogaea* L.), rich in lipids, proteins, and other nutrients, plays a pivotal role in edible vegetable oil consumption and snack food production (Burow et al. 2001; Settaluri et al. 2012; Huang et al. 2015). However, infection by *Aspergillus flavus* leads to the production of highly carcinogenic aflatoxins in seeds, undermining food safety and posing severe threats to human health (Cardwell and Henry 2004; Farombi 2006; Liao et al. 2009; Soni et al. 2020). Driven by increasing production efficiency, peanut cultivation areas have expanded continuously. Nevertheless, inadequate post-harvest drying and storage conditions in some regions have exacerbated the problem of aflatoxin

contamination. Current mitigation strategies primarily rely on physico-chemical detoxification methods (Dorner 2008; Chulze et al. 2014; Bhatnagarmathur et al. 2015). These approaches only exhibit partial effectiveness in controlling contamination; in addition, their widespread application is limited by practical challenges, high costs, and chemical residues. To date, developing and cultivating elite peanut varieties with inherent *A. flavus* resistance remains the most economically viable and sustainable strategy to eliminate or reduce aflatoxin contamination, thereby ensuring the healthy development of the peanut industry (Mehan et al. 1987; Yu et al. 2019). In this context, developing molecular markers associated with *A. flavus* resistance is crucial for accelerating breeding progress and enhancing the international market competitiveness of peanuts.

A. flavus, a common saprophytic fungus infecting peanuts, is widely distributed in soil, air, and moldy grain products. It persists in soil as conidia or sclerotia and within plant tissues as mycelium. Sclerotia primarily function as overwintering structures, enabling survival under extreme conditions such as high temperatures or drought. Conidia or ascospores produced by sclerotia facilitate dissemination and infection (Wicklow et al. 1993; Horn et al. 2009). Aflatoxins, secondary metabolites generated during mycelial growth, are classified as carcinogens (Wicklow et al. 1993; Horn et al. 2009; Yu et al. 2019). The most prevalent aflatoxins in peanuts are B₁, B₂, G₁, and G₂, with B₁ exhibiting the highest yield and the strongest toxicity (Khlangwiset and Wu 2010; Kew 2013; Caceres et al. 2020). Previous studies have indicated that resistance to *A. flavus* can occur at two distinct levels: resistance to infection and resistance to aflatoxin production. Infection resistance refers to the ability of the peanut seed coat—via its structural integrity and specific chemical composition—to prevent fungal colonization, thereby reducing or preventing aflatoxin contamination. Aflatoxin production resistance occurs when peanuts, despite being colonized by *A. flavus* in the cotyledons, support minimal or no aflatoxin synthesis by the fungus (Liao et al. 2009). These two resistance mechanisms are genetically independent (Nigam et al. 2009; Yu et al. 2019). The standard protocol for assessing infection resistance involves seed surface sterilization, rehydration, inoculation with *A. flavus*, incubation under controlled temperature and humidity, followed by quantification of spore coverage on each seed to calculate the infection index. This method is termed the in vitro seed colonization (IVSC) assay. Aflatoxin production resistance is typically evaluated by measuring aflatoxin content in inoculated seeds under laboratory conditions. Significant genotype $\times$ environment (G $\times$ E) interactions in aflatoxin contamination complicate genetic research on resistance mechanisms. The IVSC assay offers the advantage of laboratory-based evaluation, minimizing environmental interference—thus supporting its wide adoption for

preliminary screening of infection-resistant peanut germplasm and investigation of the molecular basis of resistance (Wang et al. 2016a, 2016b; Nayak et al. 2017; Korani et al. 2017; Yu et al. 2019).

Quantitative trait locus (QTL) or genome-wide association analysis (GWAS) have been successfully employed to rapidly identify molecular markers and genes associated with target traits, serving as effective tools for candidate genes mapping (Arunyanark et al. 2009; Girdthai et al. 2010; Fountain et al. 2014). In recent years, GWAS and QTL studies have been conducted in peanuts to screen for candidate regions and genes associated with resistance to *A. flavus*. QTLs associated with kernels resistance to *A. flavus* infection have been predominantly mapped to chromosomes A03, A05, A08, A10, B01, B03, B04, and B10 with 5.15%–19.00% phenotypic variance explained (PVE) (Yu et al. 2019; Khan et al. 2020; Jiang et al. 2021). Meanwhile, QTLs associated with resistance to aflatoxin production have been identified on chromosomes A01, A02, A03, A05, A07, A08, A10, B05, B06, B07, B09, and B10, with PVEs ranging from 4.61% to 21.02% (Yu et al. 2019, 2020, 2024; Jin et al. 2023). Despite these efforts, most current peanut varieties remain susceptible to *A. flavus*, therefore the identification of novel resistance sources and loci are urgently needed.

Our research group previously performed whole-genome resequencing on a population of 353 accessions with rich genetic diversity, successfully identifying genes controlling flowering pattern, testa color, and growth habit (Zheng et al. 2024). In the present study, we exploited the same resequencing data to conduct GWAS, focusing on the infection index and aflatoxin content of peanut kernels harvested from three environments after *A. flavus* inoculation. Potential candidate genes associated with *A. flavus* stress were identified by integrating annotations with findings from previous studies and haplotype analysis. The aims of our study were to (1) precisely evaluate the resistance level of 353 domestic and exotic peanut germplasm resources in response to *A. flavus* stress and identify correlations among infection index, AFB₁ and AFB₂ content; and (2) detect candidate QTLs and genes responsible for resistance to *A. flavus* stress. The QTLs identified herein can be utilized to breed cultivars with improved *A. flavus* resistance, while the detected candidate genes can undergo initial functional validation prior to their direct application in peanut genetic improvement.

Materials and methods

Plant materials and field experiment

The peanut germplasm collection previously described by Zheng et al. (2024), provided by the Henan Institute of Crop Molecular Breeding (Zhengzhou city, Henan, China), was

used in this study. This includes 353 peanut germplasm encompassing five botanical types, viz. *var. hypogaea* (185), *var. vulgaris* (128), *var. fastigiata* (26), *var. hirsuta* (12), and *var. peruviana* (2). Three control cultivars were also included in the germplasm panel: J11 (resistant to *A. flavus* infection), Zhonghua 6 (resistant to aflatoxin production), and Zhonghua 12 (susceptible to *A. flavus* infection) (Mehan et al. 1987; Cui et al. 2022). Field trials were conducted in Yuanyang (113.88° E, 35.05° N) and Shangqiu (116.12° E, 33.93° N), Henan province, during the summer seasons of 2020 and 2021, designated as CA2020 (Yuanyang, 2020), CS2020 (Shangqiu, 2020) and CS2021 (Shangqiu, 2021). Each accession was planted in two rows, with each row measuring 667 cm in length, a within-row plant spacing of 15 cm, and inter-row spacing of 50 cm. Field management followed standard agricultural practices. Seeds were harvested in batches upon maturity. Pods were sun-dried until their moisture content dropped to a safe storage level (< 10%). After shelling, the kernels were stored in seed preservation chambers (10% relative humidity, 4 °C) for subsequent analysis.

Phenotype evaluation and statistical analysis

Kernel infection indexes were assessed on materials collected across the three environments, using the method described by Khan et al (2020) with minor modifications. For each accession, 10 healthy kernels free from any apparent damage were selected. These were first immersed in 75% ethanol for 1–2 min, and then washed 5–6 times with distilled water (total washing time: 20 min) to adjust their moisture content to 15%–25%, the optimal range for *A. flavus* growth and propagation (Bhatnagarmathur et al. 2015). The highly toxigenic *A. flavus* strain As3.4408 was cultured on dichloran-glycerol-18 (DG-18) agar plates. After incubation for 7 days at 30 °C, conidia were collected and suspended in sterile water containing 0.05% Tween-80 at a concentration of 2×10^6 spores/mL. The prepared kernels were then transferred to sterilized 7-cm-diameter disposable plates, and 500 µl of *A. flavus* spore suspension was added. Plates were gently swirled to ensure full contact between the kernel surface and the spore suspension. Each plate contained 10 individually arranged kernels, and all plates were incubated in darkness at 29 ± 1 °C for 7 days, with three replicates per accession.

After incubation, resistance and susceptibility levels were scored, and the infection index was calculated according to Khan et al (2020). Specifically, the infection response was categorized into six levels: level 0: No visible green spores on kernels; level 1: 1%–10% surface covered by *A. flavus* spores, with sporadic green spores visible; level 2: 11%–20% surface covered, with distinct thick spore layers; level 3: 21%–50% surface coverage,

with thick spore layers; level 4: 51%–80% surface coverage, with patchy thick spore layers; level 5: 81%–100% surface covered, with extensive thick spore layers. The infection index was computed using the formula:

$$\text{InfectionIndex} = \frac{\sum_{i=0}^5 (w_i \times n_i) \times 100}{N}$$

w_i : Infection grade weight ($i=0$: 0%, $i=1$: 10%, $i=2$: 20%, $i=3$: 50%, $i=4$: 80%, $i=5$: 100%); n_i : the number of kernels at the i -th infection grade; N : Total number of tested kernels.

According to the infection index (II), resistance was classified as follows: HR (Highly Resistant): $\text{II} = 0$; R (Resistant): $0 < \text{II} \leq 15$; MR (Moderately Resistant): $15 < \text{II} \leq 30$; MS (Moderately Susceptible): $30 < \text{II} \leq 60$; HS (Highly Susceptible): $\text{II} > 60$.

To assess aflatoxin production in inoculated kernels, the contents of AFB₁ and AFB₂ were quantified for the CS2021 trial. Briefly, *A. flavus*-inoculated kernels were autoclaved at 121 °C for 30 min after 7 days of incubation, followed by oven-drying at 110 °C for 60 min (Fu et al. 2023; Yu et al. 2019, 2020, 2024). Briefly, 4 g of the dried sample was accurately weighed and homogenized in 40 mL of acetonitrile/water (84:16, v/v) for 10 min. Subsequently, the extract solution was purified by cleanup column and diluted for HPLC–MS analysis. Chromatographic separation was conducted by high-performance liquid chromatography (HPLC) system coupled with mass spectrometry (Shimadzu MS-8060, Kyoto, Japan). The HPLC analysis was performed at 40 °C using a C18 reversed-phase column (100 mm × 2.1 mm, 3 µm) with the mobile phase A methanol and the mobile phase B aqueous 5 mM ammonium acetate solution. A gradient elution program was conducted as the following conditions: 0 min, 70% B; 3.5 min, 20% B; 4.5 min, 20% B; 4.51 min, 70% B; 7 min, 70% B. The phase flow rate was at 300 µL min^{−1}, and 1 µL of sample was further analyzed by HPLC–MS. The multiple reaction monitoring (MRM) was used to quantify the content of AFB₁ and AFB₂. The MS parameters were set as follows: DL temperature 250 °C, heat block temperature 400 °C, nebulizing gas (N₂) 2.5 L min^{−1}, and drying gas (N₂) 10 L min^{−1}.

Descriptive statistics for infection index, AFB₁, and AFB₂ content were obtained using Microsoft Excel 2016, IBM SPSS Statistics 29.0.1.0. Phenotype correlation analysis was performed using the Performanceanalytics R package. Haplotypes analysis was conducted by Graphpad Prism 9.5. Broad-sense heritability (H^2_B) for each trait was calculated as: $H^2_B = \sigma^2_G / (\sigma^2_G + 1/r\sigma^2_e)$ (Meng et al. 2015), where σ^2_G is the genotypic variance; σ^2_e is the residual variance; r is the number of replicates (Wang et al. 2018; Shi et al. 2022).

Genome-wide association analysis

In our previous study, whole-genome resequencing was performed on a natural population comprising 353 peanut accessions at a sequencing depth of 20×. The resulting sequencing data were aligned to the cultivated peanut reference genome (Tifrunner v1.0), yielding 935,231 high-quality SNPs (Zheng et al. 2024). GWAS was conducted using three models: mixed linear model (MLM), fixed and random model circulating probability unification (FarmCPU), and Bayesian information and linkage disequilibrium iteratively nested keyway (BLINK) (Yu et al. 2006; Zhang et al. 2010; Liu et al. 2016; Huang et al. 2019) implemented in the R package GAPIT (Wang et al. 2021). Manhattan plots and Q-Q plots were generated using the R package qqman (Turner 2018). The best model was determined by comparing Q-Q plots, and the GWAS of infection index (II) and aflatoxin content were then carried out using the optimal model. The *p* value threshold was calculated using the Bonferroni method ($p \leq 0.5/935231 = 5.35 \times 10^{-7}$, $-\log_{10}(p) \geq 6.27$). SNPs with *p* value below this threshold were considered significant association locus. Phenotypic variation explained (PVE) by each significantly associated SNP was calculated based on effect error, minor allele frequency (MAF), and standard error (Shim et al. 2015).

Mining of candidate genome regions and prioritization of candidate genes via haplotype analyses

Based on the linkage disequilibrium (LD) decay estimates reported in our previous study (Zheng et al. 2024), genomic regions within 70 kb upstream and downstream of SNPs significantly associated with the traits under study were defined as candidate stable QTLs. Within the physical intervals of stable QTLs, gene models and their functional annotations were retrieved from the reference genome of the allotetraploid cultivated peanut (*Arachis hypogaea* cv. Tifrunner) (<https://www.peanutbase.org/data/v2/>) (Bertioli et al. 2019). Potential candidate genes associated with *A. flavus* resistance were subsequently predicted by integrating these annotations with findings from previous studies, and haplotype analysis was conducted on these selected candidates. Haplotype analysis was performed in three steps: First, SNP variations within the candidate genes were extracted using information from the Tifrunner reference genome (<https://www.peanutbase.org/data/v2/>) and the resequencing data (Zheng et al. 2024); second, gene haplotypes were determined based on their sequence variants; finally, materials were grouped by

the haplotype of each gene, and significance tests were conducted to assess differences between major haplotypes (Yang et al. 2023). A gene was identified as a potential candidate if significant differences were observed among its respective haplotypes.

Results

Phenotypic variation

In this study, 353 peanut accessions were artificially inoculated with *A. flavus* across three environments in Henan province, including Yuanyang in 2020 (CA2020) and Shangqiu in 2020 (CS2020) and 2021 (CS2021). The infection index of seeds exhibited extensive variation both within and across environments, ranging from 12.33 to 100.00 for CA2020, 4.33 to 96.67 for CS2020, and 2.33 to 98.67 for CS2021 (Table S1). The index followed a continuous normal distribution and fitted the normal curve well (Fig. S1), indicating that resistance to *A. flavus* infection is governed by the combined action of multiple genetic loci rather than a single major gene. Continuous distributions were observed for AFB₁ and AFB₂ contents, measured in CS2021, which ranged from 2.11 to 133.50 mg·kg⁻¹ (mean: 47.25 mg·kg⁻¹) and from 0.27 to 27.68 mg·kg⁻¹ (mean: 6.80 mg·kg⁻¹), respectively (Table S1). The broad-sense heritability (*H*²) estimates for infection index, AFB₁ content and AFB₂ content all exceeded 0.90, indicating that phenotypic variation is primarily determined by genetic factors. Furthermore, the resistance performance of the same accession remained relatively stable across environments, as evidenced by highly significant positive correlations in the infection index among the three environments (*P* < 0.001). The strongest correlation was observed between CS2020 and CS2021 (*r* = 0.67), and the weakest between CA2020 and CS2021 (*r* = 0.53) (Fig. 1). Given the highly significant positive correlations, which reflect relatively stable resistance phenotypes of individual accessions under different environmental conditions, we inferred that aflatoxin production in these accessions would exhibit similarly environmental stability. Thus, quantifying aflatoxin content in a single representative environment is sufficient to characterize the inherent aflatoxin-related phenotypic traits of the accessions.

Thirteen accessions displayed stable resistance across the three environments, with infection index < 30 (Table 1). Their AFB₁ content ranged from 2.11 to 28.95 mg·kg⁻¹, which were significantly lower than that of Zhonghua 6—the aflatoxin-resistant control cultivar, indicating these accessions possess dual resistance to *A.*

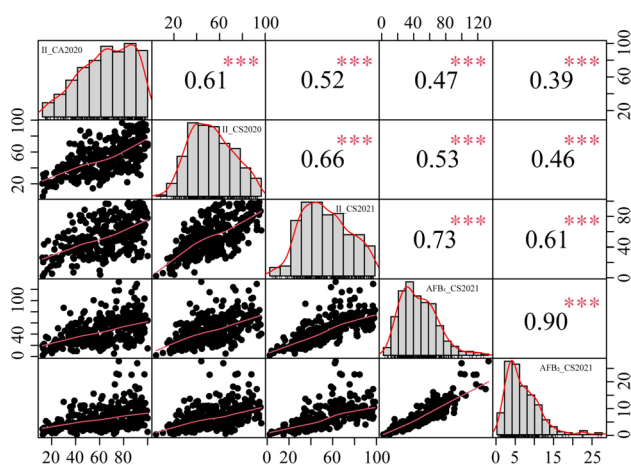


Fig. 1 Phenotypic correlations among infection index, AFB₁ and AFB₂ content in 353 peanut accessions. ***: significant correlated at the 0.001 level ($P \leq 0.001$); II_CA2020: infection index at Yuanyang in 2020; II_CS2020: infection index at Shangqiu in 2020; II_CS2021: infection index at Shangqiu in 2021. AFB₁_CS2021: AFB₁ content at Shangqiu in 2021; AFB₂_CS2021: AFB₂ content at Shangqiu in 2021. The same as below

flavus infection and aflatoxin production. Among them, accessions C203 and C206 performed exceptionally resistance, characterized by infection index as low as 2.33 and 6.00, and AFB₁ contents of 2.11 and 6.49 mg·kg⁻¹, respectively. In the CS2021 environment, the infection index showed highly significant positive correlations with AFB₁ and AFB₂ contents ($r = 0.73$ and 0.62 , $P < 0.001$) (Fig. 1). Additionally, a strong positive correlation was observed between AFB₁ and AFB₂ contents ($r = 0.90$, $P < 0.001$), suggesting that higher infection levels are typically associated with greater aflatoxin accumulation, and the synthesis

of these two aflatoxins may be regulated by shared genetic mechanisms.

Comparative analysis of infection index and aflatoxin content among peanut germplasm of different botanical and growth habit types

We compared infection index and aflatoxin (AFB₁, AFB₂) contents among four peanut botanical types (*var. fastigiata*, *var. hirsuta*, *var. hypogaea*, and *var. vulgaris*) and three different growth habits (erect, semi-prostrate, prostrate). As shown in Fig. 2A, *var. hypogaea* showed a significantly wider variation range in infection index than the other three types, with a lower median value, indicating that this type is more likely to yield materials resistant to *A. flavus* infection. The median contents of AFB₁ and AFB₂ in *var. hypogaea* were significantly lower than those in *var. fastigiata* and *vulgaris*, suggesting its greater potentials also in terms of resistance to aflatoxin production. Consistent with this, among the 13 accessions identified as resistant, 12 belong to *var. hypogaea*, suggesting that the botanical type may serve as auxiliary reference indicator in resistance screenings.

Erect-type accessions exhibited significantly larger variation in infection index, AFB₁ and AFB₂ content compared with prostrate-type accessions (Fig. 2B). However, prostrate-type germplasm had a lower median value, suggesting that this kind of material may contain a higher proportion of resistance sources. In accordance, prostrate and semi-prostrate accessions—accounting for about 27% of the total germplasm collection—were associated with about 54% (7 out of 13) of the resistant accessions. Taken together, this indicates that the growth habit may also be considered as an indicator in resistance screening.

Table 1 Features of 13 peanut accessions with both infection and aflatoxin production resistance

Accession number	II_CA2020	II_CS2020	II_CS2021	AFB ₁ _CS2021	AFB ₂ _CS2021	Botanical type	Growth habit	Region
C115	20.33	19.00	26.67	15.36	2.13	<i>var. hypogaea</i>	Erect type	Henan, China
C130	23.33	16.00	16.33	28.95	4.07	<i>var. hypogaea</i>	Semi-prostrate type	Hebei, China
C172	16.67	16.33	11.00	10.85	1.68	<i>var. hypogaea</i>	Erect type	Henan, China
C174	23.33	24.00	25.50	12.32	0.91	<i>var. vulgaris</i>	Erect type	India
C203	12.67	4.33	2.33	2.11	0.36	<i>var. hypogaea</i>	Prostrate type	Henan, China
C206	14.00	10.33	6.00	6.49	0.97	<i>var. hypogaea</i>	Erect type	Henan, China
C220	28.00	23.00	22.78	26.58	5.30	<i>var. hypogaea</i>	Erect type	Henan, China
C270	23.67	17.33	26.67	25.78	2.08	<i>var. hypogaea</i>	Erect type	Henan, China
C273	20.33	17.00	5.33	6.08	0.83	<i>var. hypogaea</i>	Semi-prostrate type	America
C331	14.33	27.00	25.50	10.31	2.19	<i>var. hypogaea</i>	Semi-prostrate type	Hubei, China
C344	20.67	18.67	23.00	21.08	3.57	<i>var. hypogaea</i>	Prostrate type	India
C354	12.33	20.33	26.33	19.27	2.40	<i>var. hypogaea</i>	Prostrate type	America
C368	19.33	22.00	15.00	18.00	3.23	<i>var. hypogaea</i>	Semi-prostrate type	Shandong, China

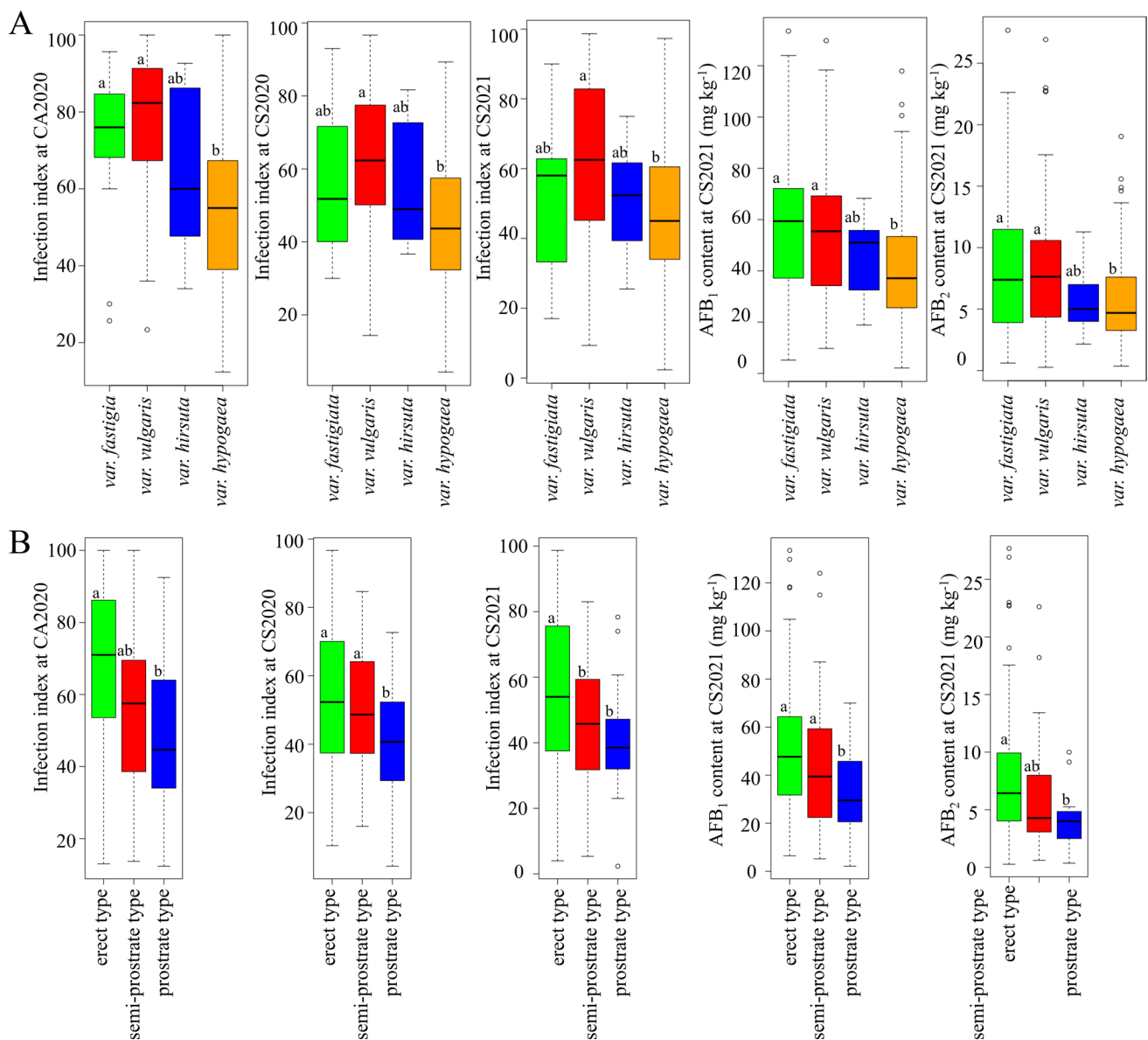


Fig. 2 Box plot of infection index, AFB₁ and AFB₂ content in different botanical types (A) and growth habit types (B) of peanuts. Different letters indicate the significant difference at $P \leq 0.05$ (one-way ANOVA)

Genome-wide association study

Genome-wide association analyses (GWAS) were performed for the infection index, as well as AFB₁ and AFB₂ contents, by integrating resequencing and phenotypic data in the germplasms using three models. MLM, the best model among the three tested models according to the results of QQ plots (Fig. S2), were selected for subsequent GWAS analysis. For the infection index, five significant SNPs were detected in the CA2020 environment, distributed on chromosomes A02, A05, and B03, with phenotypic variation explained PVEs ranging from 7.98% to 8.41% (Fig. 3, Table S2). Two significant

SNP associations were identified in CS2021, located on chromosomes A05 and B08, while no significant SNPs were detected in CS2020. Overall, one SNP was stably associated with infection index across at least two environments (Fig. 3), with the PVE ranging from 7.91% to 8.13% (Table 2, Table S2). Regarding aflatoxin contents, three significant SNPs were associated with AFB₁ (distributed on chromosomes A03, A05, A08), and three significant SNPs were linked to AFB₂ content (distributed on chromosomes A03, A05 and B05) with PVEs of 7.69% to 9.57% (Fig. 3, Table S2). Notably, 2 SNPs (A03_14418931 and A05_91706320) were significantly associated with both AFB₁ and AFB₂ contents ($r = 0.90$,

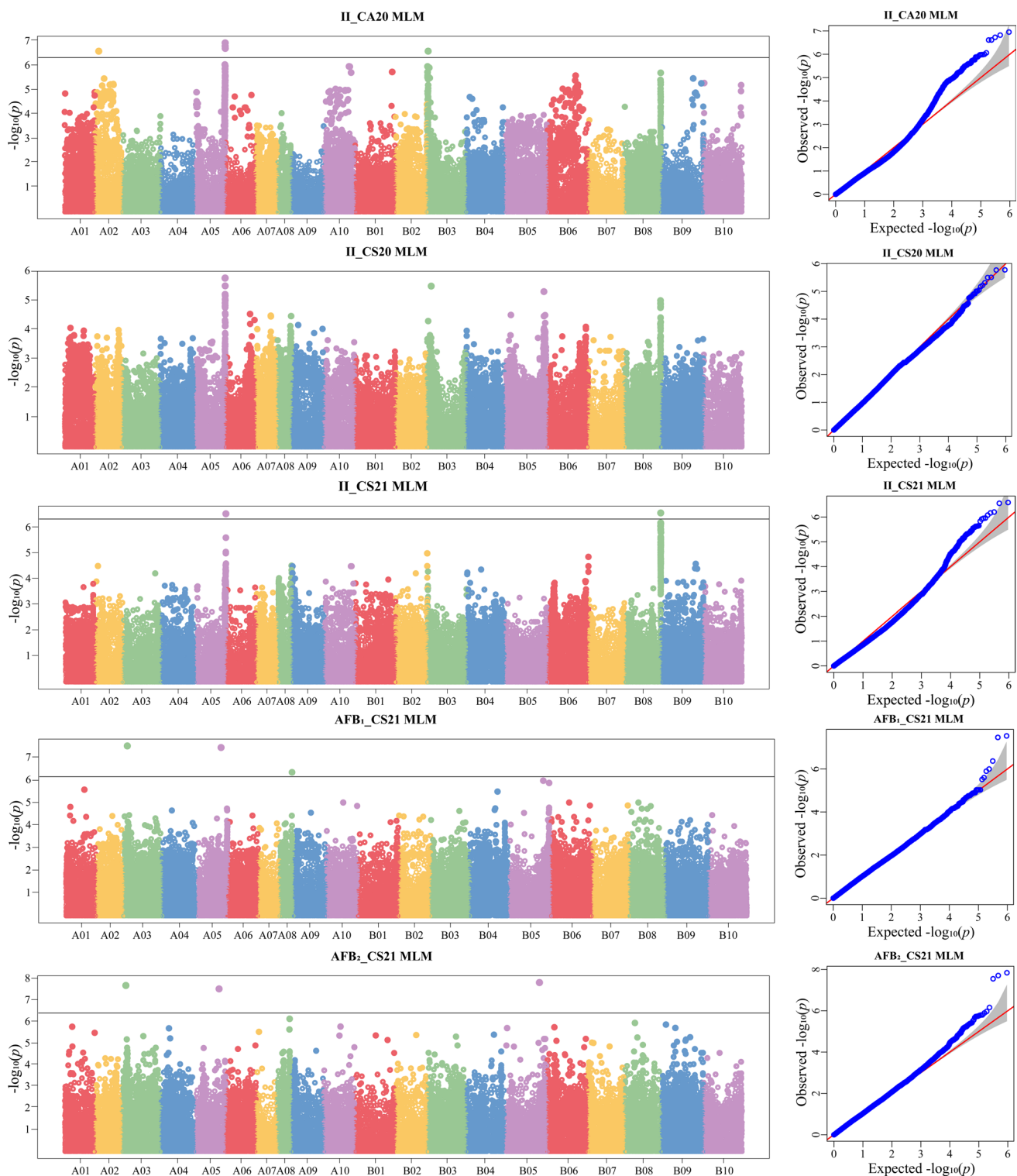


Fig. 3 The manhattan and QQ-plots based on MLM module

$P < 0.001$) (Table S2), suggesting potential genetic correlation or pleiotropic effects in the synthesis of these two aflatoxins. Furthermore, the SNP signal associated with infection resistance were physically distant from the

three aflatoxin production-related SNPs, supporting the notion that infection resistance and aflatoxin production resistance are independently inherited traits.

Table 2 The significant SNPs associated with resistance to *A. flavus* infection and aflatoxin production of peanut kernels

SNP	Chr	Position	Allele	$-\log_{10}(P)$	Env	PVE (%)
A05_115249952	A05	115,249,952	C/T	6.72	II_CA20	8.13
				6.56	II_CS21	7.91
A03_14418931	A03	14,418,931	A/-	7.53	AFB ₁ _CS21	9.18
				7.71	AFB ₂ _CS21	9.40
A05_91706320	A05	91,706,320	G/A	7.46	AFB ₁ _CS21	9.09
				7.55	AFB ₂ _CS21	9.20

Table 3 Three stable candidate QTLs identified for resistance to *A. flavus* stress

QTLs	Key SNP	Chromosome	Start	End	Length (kb)
<i>qII_A05</i>	A05_115249952	5	115,179,952	115,319,952	140
<i>qAF_A03</i>	A03_14418931	3	14,348,931	14,488,931	140
<i>qAF_A05</i>	A05_91706320	5	91,636,320	91,776,320	140

Classification of stable QTLs and identification of candidate genes for *A. flavus* resistance

Based on previous LD decay estimates (Zheng et al. 2024), initial QTLs were defined as 70 kb intervals flanking significantly associated SNPs. One SNP stably associated with infection resistance in at least two environments and two SNPs associated with both AFB₁ and AFB₂ defined three QTLs on chromosome A03 and A05, named *qII_A05*, *qAF_A03*, and *qAF_A05*, respectively, all spanning 140 kb (Table 3). These QTLs/genetic elements exhibited pronounced signal peaks and distinct haplotype differentiation, suggesting the potential presence of key genes within these regions that regulate peanut response to *A. flavus* stress. A total of 41 genes were annotated within the physical intervals of the three stable QTLs. By integrating functional annotations from the peanut reference genome and findings from existing scientific literature, 13 genes potentially associated with *A. flavus* resistance were initially selected (Table S3). Sequence variation analysis of these 13 candidate genes was performed using resequencing data from 353 peanut germplasms, with three genes exhibiting distinct sequence variations. These genes are as follows: *Arahy.7046BI.1*, encoding a protein containing both a pleckstrin homology (PH) domain and a lipid-binding START domain; *Arahy.TX2FLU.1*, encoding a receptor-like kinase; and *Arahy.ASR4JM.1*, encoding a disease resistance protein of the TIR-NBS-LRR class. Each of these three genes harbored two distinct haplotypes, with all exhibiting significant associations between haplotype variation and *A. flavus* resistance traits—of which two were linked to infection resistance and one to aflatoxin production resistance (Fig. 4, Table S4).

For the two candidate genes associated with *A. flavus* infection resistance (*Arahy.7046BI.1* and *Arahy.TX2FLU.1*), haplotype analysis revealed that peanut accessions carrying

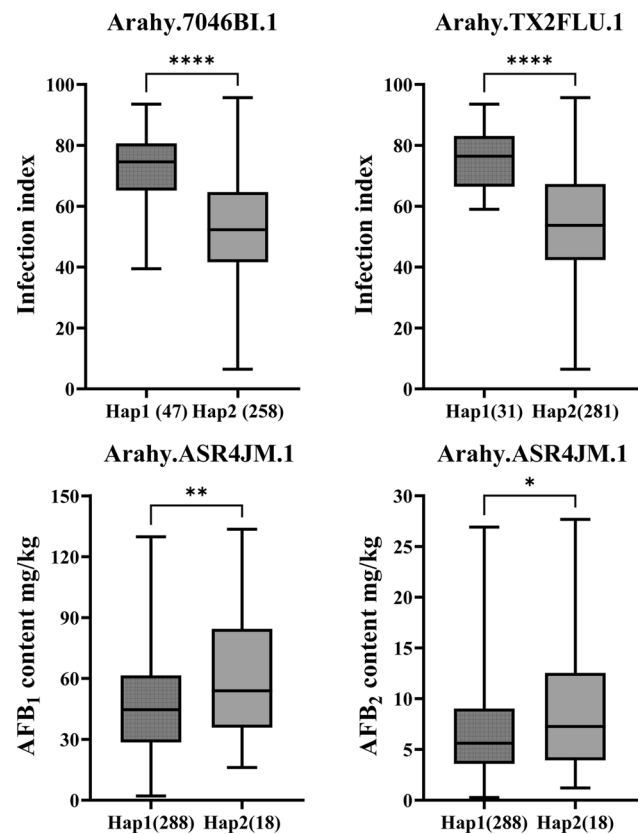


Fig. 4 Haplotype analysis of 3 candidate genes whose haplotype alleles showed significant differences in the regulation of response to *A. flavus* stress. The significance of differences between different haplotypes was indicated by “*” at 0.05 level, “**” at 0.01 level, “***” at 0.001 level, “****” at 0.0001 level by ANOVA

Hap2 exhibited an average reduction in infection index of 27.14% and 27.17%, respectively, compared with those carrying Hap1—indicating that accessions with Hap2 display enhanced *A. flavus* infection resistance. To quantify

the strength of these haplotypic effects, we calculated the standardized effect size (Hedges' g based on independent samples t test) and found values of -2.49 and -2.40 for Hap2 of the two genes, respectively, corresponding to very large effects in plant quantitative genetics ($|g| \geq 2.0$, indicating a strong regulatory role in resistance). Regarding haplotype frequency in germplasm, based on statistics from tested peanut accessions, Hap2 (the resistance-favorable haplotype) accounted for 85.43% and 90.6% of the total accessions, respectively. These results indicate that the favorable haplotypes are relatively common in the current germplasm pool, representing widely available and valuable genetic resources for resistance breeding (easy to utilize in cross-breeding due to their high frequency). In addition to aflatoxin production resistance, we also analyzed the haplotype effects of the candidate gene associated with aflatoxin production resistance (Arahy.ASR4JM.1): Accessions harboring Hap1 showed an average decrease of 26.68% in AFB₁ content and 28.93% in AFB₂ content relative to those with Hap2, demonstrating that accessions carrying Hap1 possess superior resistance to aflatoxin production. The standardized effect size (Hedges' g) of Hap1 for AFB₁ and AFB₂ content was -0.72 and -0.62 , respectively (moderate effects, $|g| = 0.5\text{--}0.8$). Frequency statistics from the tested accessions showed that Hap1 (the aflatoxin resistance-favorable haplotype) was present in 94.12%, suggesting it is a high-frequency favorable haplotype with substantial potential for direct utilization in aflatoxin-resistant peanut breeding.

Discussion

Peanut (*Arachis hypogaea* L.) is highly susceptible to *A. flavus* infection, which produces highly carcinogenic aflatoxins, posing severe food safety risks and hindering the sustainable development of the peanut industry (Cardwell and Henry 2004; Farombi et al. 2006; Liao et al. 2009). Cultivating resistant varieties remains the most economically viable and sustainable strategy to mitigate aflatoxin contamination, yet most current varieties lack sufficient resistance (Mehan et al. 1987; Yu et al. 2019). In this study, we evaluated 353 peanut accessions via in vitro artificial inoculation, observing stable resistance performance across environments (Fig. 1). This stability, coupled with high broad-sense heritability (>0.90), validates the reliability of our phenotypic evaluations for genetic mapping.

Germplasm with low infection index as priority for screening aflatoxin production resistance

A strong positive correlation ($r=0.73$, $P<0.001$) was found between infection index and AFB₁ content, alongside the absence of accessions with low infection index but high

aflatoxin accumulation, indicates prioritizing low infection index germplasm can efficiently screen for aflatoxin production resistance. The superior accessions identified in our study, C203 and C206, represent a desirable “dual-resistance” type, inhibiting both fungal colonization and aflatoxin synthesis—more practical for breeding than the “high infection but low aflatoxin” type exemplified by Zhonghua 6 (Wang et al. 2016a). Additionally, the high correlation between AFB₁ and AFB₂ ($r=0.90$, $P<0.001$) and AFB₁'s high toxicity (Kew 2013; Caceres et al. 2020) support AFB₁ as a key indicator for aflatoxin resistance evaluation.

Strengthen screening and evaluation of *A. hypogaea* and prostrate-type peanut germplasm

Associations were observed between germplasm categories and response to *A. flavus*, with lower infection index and aflatoxin (AFB₁ and AFB₂) content in *var. hypogaea* or prostrate-type peanuts. In more details, among the 13 resistant accessions identified in this study, 12 belong to *var. hypogaea*, and semi-prostrate/prostrate types (27% of the panel) accounted for 54% of resistant accessions. This suggests artificial selection for erect growth habits may have reduced some resistance-related genetic resources, while *var. hypogaea* retained inherent resistance potential during domestication. Based on these observations, future *A. flavus* resistance breeding may benefit from prioritizing *var. hypogaea* and prostrate-type germplasm to efficiently enrich resistance resources, while integrating valuable resistance sources from other botanical types (e.g., *var. fastigiata* for short-life-cycle regions) to develop regionally adapted elite varieties.

Novel QTL and genes associated with resistance to *A. flavus* stress

Genome-wide association study (GWAS) is a powerful tool for identifying genes associated with complex traits (Cockram et al. 2010; Chia et al. 2012; Chen et al. 2014; Liu et al. 2025). Yu et al. (2020) reported the results of a GWAS conducted using 99 peanut accessions, resulting in the identification of 60 SNPs, distributed on chromosomes A03, A05, A07, A08, A10, B02, B04, B05, B06, B07, B08, B09, and B10, with 26.87%–31.70% PVE. In recent years, researchers have mapped *A. flavus* infection resistance and aflatoxin production resistance using recombinant inbred line (RIL) populations. Specifically, QTLs associated with infection resistance have been localized to peanut chromosomes A03, A05, A08, A10, B03, B04, and B10, while those linked to aflatoxin production resistance have been identified on chromosomes A05, A07, and B06 (with PVEs ranging from 3.64% to 19.04%). Notably, chromosomes A07 and B06 are consistently reported as major hotspots for aflatoxin

resistance QTLs/genes across independent studies, reflecting a conserved genetic basis underlying this agronomically important trait. However, most candidate genes lack functional validation, with only *AhAtr1* (Yu et al. 2024) functionally characterized and applied in breeding. In this study, GWAS using the MLM model identified 10 significant SNP-trait associations, including 1 stable SNP for infection resistance and 2 SNPs co-associated with AFB₁/AFB₂ production. These SNPs delineated three novel stable QTLs (*qII_A05*, *qAF_A03*, *qAF_A05*) on chromosomes A03 and A05 (Table S3), distinct from previously reported loci (Yu et al. 2019, 2024; Khan et al. 2020; Jiang et al. 2021; Jin et al. 2023), expanding the genetic resource pool for peanut resistance breeding.

Within these QTLs, three genes (encoding PH/START domain-containing protein, receptor-like kinase, and a TIR-NBS-LRR class disease resistance protein) were validated via haplotype analysis. For the PH/START domain-containing protein, its *Arabidopsis* homolog receptor-like kinase EDR2 acts as a negative regulator of powdery mildew resistance dependent on salicylic acid (SA) signaling, with conserved homologs across rice and barley (Tang et al. 2002, 2005). In chickpea, START domain-containing genes exhibit conserved lipid binding/transport functions and are induced by drought, salt, and wounding (Satheesh et al. 2016). These findings support the peanut candidate gene's potential involvement in conserved lipid-mediated defense pathways. Receptor-like kinase (RLKs) function as pattern recognition receptors (PRRs) to perceive pathogen-associated molecular patterns (PAMPs) and trigger basal defense (PTI) (Boller and Felix 2009; Zipfel et al. 2014; Wang et al. 2016c). TIR-NBS-LRR proteins, core components of effector-triggered immunity (ETI), recognize pathogen effectors to activate enhanced immune responses (Jones and Dangl 2006; Cui et al. 2015). Although PTI/ETI responses in *A. flavus*-infected peanut kernels have not been explicitly observed, the identification of these immune-related candidate genes suggests complex defense mechanisms blocking fungal invasion and proliferation. In summary, this study provides insights into the genetic basis of *A. flavus* resistance in peanut, identifies novel resistant germplasm, QTLs, and candidate genes, and offers valuable resources for accelerating resistance breeding.

Conclusion

This study systematically evaluated 353 peanut accessions for resistance to *Aspergillus flavus* infection and aflatoxin (AFB₁, AFB₂) production across three environments, identifying 13 accessions with stable dual resistance, including the outstanding lines C203 and C206. Correlation analysis

confirmed significant positive links between infection index and aflatoxin contents. Notably, *var. hypogaea* (12 out of 13 resistant accessions) and prostrate/semi-prostrate accessions (54% of resistant lines despite accounting for 27% of the panel) were enriched in resistance resources, providing targeted directions for germplasm exploitation. GWAS using the mixed linear model (MLM) identified 10 significant SNP-trait associations, including 1 stable SNP for infection resistance (across ≥ 2 environments) and 2 SNPs co-associated with both AFB₁ and AFB₂ production. Based on linkage disequilibrium (LD) decay estimates (70 kb upstream/downstream of significant SNPs), three novel stable QTLs (*qII_A05*, *qAF_A03*, *qAF_A05*) were delineated on chromosomes A03 and A05, distinct from previously reported loci. Haplotype analysis validated three candidate genes within these QTLs: *Arahy.7046BI.1* (encoding a PH/START domain-containing protein), *Arahy.TX2FLU.1* (encoding receptor-like kinase), and *Arahy.ASR4JM.1* (encoding TIR-NBS-LRR class disease resistance protein). These genes exhibited significant haplotype-trait associations, with favorable haplotypes showing high frequencies (85.43%–94.12%) in the germplasm pool, facilitating their direct utilization in marker-assisted breeding. In summary, this study uncovers key genetic loci and candidate genes underlying peanut resistance to *A. flavus* infection and aflatoxin production, provides elite dual-resistant germplasm, and clarifies targeted germplasm screening strategies. The findings not only enrich our understanding of the genetic architecture of *A. flavus* resistance in peanut but also lay a solid foundation for further functional validation of candidate genes and the development of molecular markers.

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Author contribution statement MJ C, SY H, and XY Z conceived and designed the experiment. MJ C, JK G, LJ C, and Z W conducted experiments and performed data analysis. MJ C wrote the manuscript. FY Q, ZQ S, XR X, XB W, and M Z performed phenotypic evaluation. BY H, WZ D, SY H, and ZY Z drafted proposals and corrected the manuscript. All authors read the manuscript and approved it for publication.

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Data availability Data presented in this study are available in this research article and supplementary materials.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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